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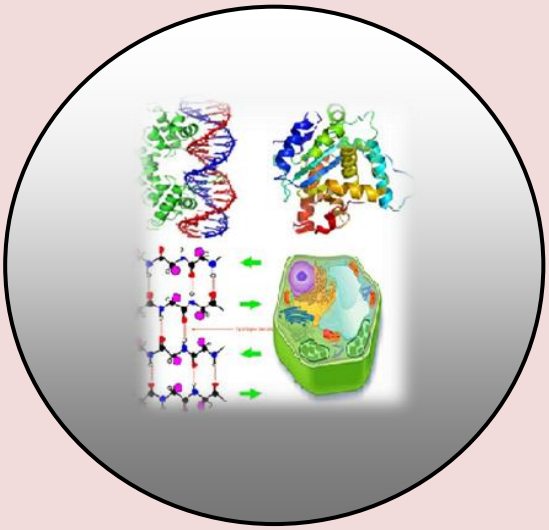

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*Syzygiumaromaticum*Silver Nanorods- A Promising Molecule to Control Multidrug-resistant *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Present study investigated the reducing and capping ability of Syzygiumaromaticum to synthesize nanoparticles. These nanoparticles were further tested to control multidrug resistant bacterial species. In a result S. aromaticum formed nanorods at pH 9.5 and these were more efficient in controlling E. coli and S. aureus with zone of inhibition recorded maximum as 18mm and 31 mm, respectively. In a conclusion, S. aromaticumnanorods certainly recorded with promising features and represented as future drug to better control human pathogens especially E. coli and S. aureus.

Keywords: Silver nanoparticles, Syzygiumaromaticum, antibacterial activity, drug resistance control.

INTRODUCTION

Research is progressing towards nanoworld to discover nanoparticles originated from the plant, animal and microbes that promises uncountable potential in many applications (Husen and Siddiqui, 2014; Siddiqi and Husen, 2016).

In a view, research is much focused on silver nanoparticles which represent its unique feature to control bacterial growth by regulating them once present in an environment (Wei et al., 2015; Lara et al., 2011; Siddiqi and Husen, 2016).

In the present time plants are reported to produce AgNPs capable of controlling the number of pathogenic bacteria for example., *Piper longum* (Reddy et al., 2014)*; Aloe vera* (Logaranjan et al., 2016). As per the present study,*Syzygiumaromaticum* known as clove found to be rich in bioactive compounds such as flavonoids, hydroxybenzoic acid, hydroxicinnamic acid and others important for its bioactivity (Neveu et al., 2010). Not only is that clove remaining abundant by production in countries like India, Indonesia, Malaysia, Sri-Lanka, Madagascar and Tanzania (Kamatou et al., 2012). Keeping in mind, all these aspects in the present study antibacterial features of the prepared AgNPs from *S. aromaticum*flower bud has investigated in detail especially against *E. coli* and *S. aureus* recognised as multidrug-resistant human pathogens.

MATERIAL AND METHODS

In the present study ability of silver nanoparticles (AgNPs) synthesized from *Syzygiumaromaticum*flower bud as an antibacterial agent was carried out. Synthesized AgNPs checked against *E. coli* and *S. aureus* isolated from blood, pus and urine samples of human patients when tested under *in vitro* conditions.

a) Sampling of bacterial pathogens

Human patients were visiting local pathology laboratories consulted to donate blood, pus and urine samples for investigation. These samples were inoculated on selective and differential media such as Eosin methylene blue and Mannitol salt agar to isolate *E. coli* and *S. aureus* species, respectively by incubating plates at 37℃ for 24 hours. Appeared colonies were sub-cultured and purified for further analysis.

b) Antibiotic Sensitivity Assay

Once *E. coli* and *S. aureus* isolates identified those were checked for antibiotic sensitivity by using Kirby Bauer antibiotic testing method. Here 16 antibiotics were tested such as Azlocillin (AZ 75), Doripenem (DOR 10), Cefotaxime (CTX 30), Amikacin (AK 30), Ticarcillin (Ti 75), Imipenem (IPM 10), Polymyxin B (PB 300), Levofloxacin (LE 5), Cefoperazone (CPZ 75), Ceftriaxone (CTR 30), Piperacillin/Tazobactam (PIT 100/10), Norfloxacin (NX 10), Aztreonam (AT 30), Carbenicillin (CB 100), Mezlocillin (MZ 75) and Netillin (NET 30). Plates incubated at 37℃ for 24 hours and zone of inhibition in millimetre compared as per CLSI chart to detect drug-resistant strains.

c) Synthesis of *S. aromaticum*AgNPs

In a processing market available *S. aromaticum*flower buds (10 grams) washed in distilled water two-three times. These buds sterilised using 0.1% $HgCl_2$ for one minute and washed with sterile distilled water to remove microbial contaminants. Sterilised buds were crushed to homogenise and suspended in 100ml of sterile distilled water. The solution then kept for boiling at 60℃ for 15 minutes. Once boiled, solution kept on cooling and filtered by Whatmann No. 1 filter. Filtrate stored at 4℃ until further use. The prepared filtrate of clove bud (10 ml) added in 90 ml of 1mM silver nitrate solution prepared in distilled water and incubated for 24 hours to confirm the formation of AgNPs via spectrophotometry.In a confirmation process, absorption maxima recorded at the wavelength range of 360-700 nm when sample matured at 24 hrs.

In a similar pattern, changes in the medium pH made such as 3.5, 4.5, 5.5, 7.5 and 9.5 to synthesize nanoparticles. In a second set by setting pH at 5.5, temperature based AgNPs synthesized from clove buds. Lastly in a reducing agent set glucose was added as 1%, 2%, 3% and 4% to synthesize AgNPs from clove buds.In all sets of studies, formed AgNPs confirmed by UV-Visible spectrophotometer using wavelength range 360-700 nm.

d) Antibacterial activity of AgNPs

Antibiotic-resistant *E. coli* and *S. aureus* recorded for growth inhibitory possibility by involving variable concentrations of AgNPs.Here formed AgNPs centrifuged to obtain pellet at 10,000 g. Pellet then suspended in 1.5 ml of 0.9% DMSO to prepare a homogenous solution.

In an antibacterial assay, nutrient agar plates inoculated with bacterial isolate with the inoculum (100 µl) set as 0.5 McFarland optical density. It was then punctured with wells and loaded with four concentrations of AgNPs (25, 50, 75 and 100 µl). Plates were incubated at 37℃ for 24-48 hours and formed zone of inhibition was recorded.

Characterisation of AgNPs

The most effective AgNPs formed at given condition was characterised for features by involving Scanning Electron Microscopy and determined at the nanoscale for frequency, shape and size of AgNPs.

RESULTS

a) Isolation of *E. coli* and *S. aureus*

The present study investigated pus, blood and urine samples of human patients by inoculating samples on Eosin methylene blue and Mannitol salt agar. Result showcased these samples did showcase the presence of *E. coli* and *S. aureus* based on selective media growth as in Table 1 and Fig. 1 recorded as CFU.

b) Antibiotic sensitivity assay

As per Kirby Bauer antibiotic sensitivity report when isolate *E. coli* strains (n=10) tested for 16 antibiotics; 50% *E. coli* recorded multidrug resistance to antibiotics (≥5) while other 50% strains with a minimum of 2 antibiotics. Among the strains, 60% of them are strongly resistant to Cefotaxime and 50% to Imipenem. In contrast antibiotics, Ceftriaxone and Netillin reported only 10% and 0% resistance, respectively in *E. coli* as in Table 2 and Fig. 2.

In a similar study, *S. aureus* 70% strains (n=7) recorded multidrug resistance ≥5 antibiotics out of 16 antibiotics tested. Among those most resistant antibiotics recorded as Cefotaxime (60%), Ceftriaxone (60%) and Ticarcillin (50%). In contrast, Piperacillin/Tazobactam, Netillin and Norfloxacin reported the least (10%) resistance as in Table 3 and Fig. 3.

c) Formation of AgNPs

S. aromaticum(clove) flower bud reduced silver nitrate (1mM) successfully in all sets of parameters as evident by the browning of clove plus $AgNO_3$ solution after 24 hours of incubation as in Fig. 4. Synthesized AgNPs also formed typical absorption maxima at 440 nm when tested by UV-Vis spectroscopy as in Fig. 5.

d) Antibacterial activity of AgNPs

*S. aromaticum*clove buds AgNPs prepared at different pH, temperature and glucose recorded to form varied AgNPs as indirectly evident from different antibiotic sensitivity lies with them. Here MDR *E. coli* strains reported the most sensitive towards AgNPs formed at pH 9.5 with 12-18 mm of inhibition.

In the case of MDR *S. aureus*AgNPs formed at pH 9.5 once again able to showcase zone of growth inhibition from 18-31 mm in 66% of strains.In all other sets, we did not record any growth inhibition worth consideration as shown in Table 4.

e) Scanning Electron Microscopy

As from the study *S. aromaticum*flower buds, AgNPs formed at pH 9.5 recorded to be better in performance; we studied its shape, size and architecture. As per SEM analysis, pH 9.5 based clove AgNPs showcased nano-rod shape with 4000-12000 nm in length and about 400-1200 nm in width as shown in Fig. 6.

DISCUSSION

Plants always come to rescue a human from troubles they created on their own. In one such study, earlier human developed the antibiotics to control the range of bacterial pathogens but in recent time failed to control developing drug resistance mechanism in them. Such resistance has undoubtedly increased the mortality and morbidity in patients with MDR pathogens and demand urgent attention.

In the present study, we isolated known human pathogens as *E. coli* and *S. aureus* remained localised in pus, blood and urine. These isolates were confirmed to represent multidrug resistance as per CLSI standards.

In a remedy by producing Silver nanoparticles from plant *S. aromaticum* flower bud, we successfully reported the ability of AgNPs of clove bud able to control these MDR pathogens.

In a series of testing, we developed AgNPs by variation in pH, temperatureor reducing sugar concentration. Here the best AgNPs received to be antibacterial (*E. coli* as well as *S. aureus*)that of pH 9.5 with 12-31 mm of inhibition controlling *S. aureus* as well as *E. coli*.

In a continuing study, AgNPs characterised by SEM as nanorods those found to be controlling *S. aureus* and *E. coli* when formed at pH 9.5.

In a similar study, Ahmad et al., (2015) reported the multidrug resistant *E. coli* sampled from Urinary tract infection. Salem et al., (2016) reported that *S. aureus* in the large number (n=281) isolated successfully with MDR features on urine cultures.

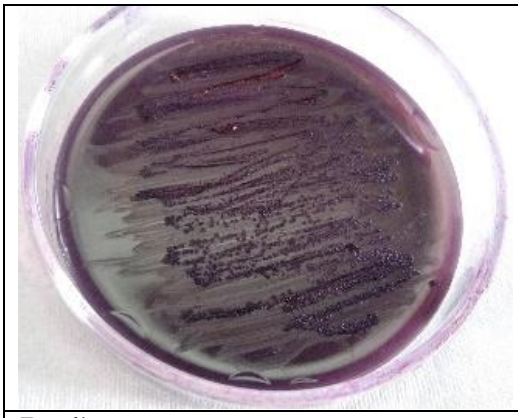
The present study reported *E. coli* strains resistance towards ampicillin, cefotaxime, sulfamethoxazole, aprofloxacin and trimethoprim with 50% strains resistance towards ≥5 antibiotics.

The study also demonstrated *S. aureus* with 70% strains with ≥5 antibiotics resistant and with 60% resistance towards Ceftriaxone and Cefotaxime followed by 50% for Ticarcillin.

In a remedy, we proposed to control these MDR pathogens using AgNPs derived from clove buds especially at basic pH 9.5.

Table 1. Colonies retrieved from Urine, Blood and Pus on selective media.

	E.M.B.	M.S.A.
Urine	15	12
Blood	7	5
Pus	15	16
EMB: Eosin methylene blue agar; MSA: Mannitol salt agar		



E. coli

S. aureus

Figure 1. Cultural Morphology recorded for the *E. coli* and *S. aureus* on EMB and MSA agar.

Table 2. Antibiotic activity against *E. coli.*

Sr.No.	Antibiotics	Abbreviations	Zone of Inhibition (mm)									
			E. coli 1	*E. coli 2*	*E. coli 3*	*E. coli 4*	*E. coli 5*	*E. coli 6*	*E. coli 7*	*E. coli 8*	*E. coli 9*	*E. coli 10*
1	Azlocillin	AZ75	S	S	S	S	S	S	R	S	S	S
2	Doripenem	DOR10	S	S	R	S	R	S	S	S	S	S
3	Cefotaxime	CTX30	R	S	S	S	R	S	R	R	R	R
4	Amikacin	AK30	S	S	S	S	S	S	S	S	R	R
5	Ticarcilin	TI75	R	R	R	S	S	S	S	S	R	S
6	Imipenem	IPM10	R	S	S	R	R	S	R	R	S	S
7	Polymixin-B	PB300	R	S	R	S	S	S	S	S	S	S
8	Levofloxacin	LE5	S	R	S	R	S	S	R	S	S	R
9	Cefoperazone	CPZ75	S	S	S	S	R	S	S	R	S	R
10	Ceftriaxone	CTR30	R	S	S	S	S	S	S	S	S	S
11	Piperacillin/Tazobactam	PIT100/10	S	S	R	S	S	S	R	S	S	S
12	Norfloxacin	NX10	S	R	S	S	R	S	S	S	S	S
13	Aztreonam	AT30	R	S	S	S	S	S	R	S	S	S
14	Carbenicillin	CB100	R	S	S	S	R	S	R	S	S	R
15	Mezlocillin	MZ75	R	S	S	S	R	S	S	S	S	S
16	Netillin	NET30	S	S	S	S	S	S	S	S	S	S
		resistant	8	3	4	2	7	0	7	3	3	5
		sensitive	8	13	12	14	9	16	9	13	13	11
S: Sensitive; R: resistance as per CLSI chart												

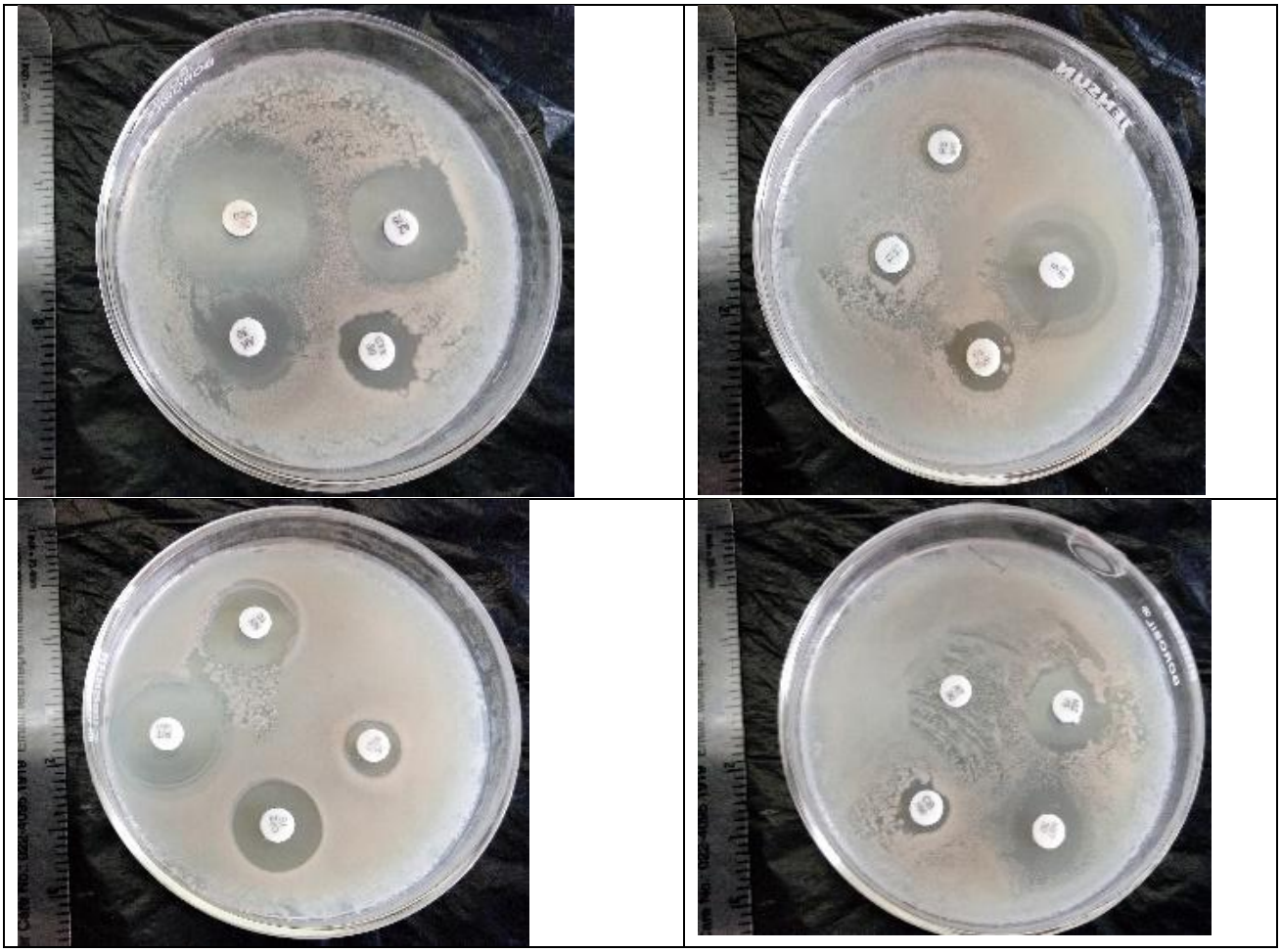


Figure 2. Antibiotic sensitivity pattern of *E. coli.*

In similar studies, Phanjom and Ahmed (2017) synthesized AgNPs at pH 4-10 in the presence of silver nitrate when treated with cell filtrate of *Aspergillus oryzae*. They also reported that using primary AgNPs antibacterial activity increases many folds. Here we observed that AgNPs formed at different pH(3.5, 4.5, 5.5, 7.5 and 9.5) must have different shapes and sizes since each group recorded with a different zone of inhibition against the bacterium. In a similar report Alqadi et al., (2014) elaborated on changing pH in the presence of ascorbic acid reported to pH changes in morphology of nanoparticles and its linked activity.

Here in the study, MDR *E. coli* get inhibited with AgNPs formed at pH 9.5 with 12 mm of inhibition. In a similar study, Su et al., (2017) reported a likely effect of AgNPs when decorated with lipase sensitive polyurethane micelles able to control *E. coli* and *S. aureus* by degradation of the polymer matrix.

In a study, MDR *S. aureus* successfully growth controlled with pH 9.5 with the best zone of inhibition of 31 mm which was superior to any antibiotic tested.

Kumari et al., (2017) advocated using silver nanoparticles to control *E. coli,S. aureus, S. marcescens, P. aeruginosa* and *Shigellasonnei.* They reported *S. aureus* 53.9% of strains got successfully controlled in the presence of AgNPs. Su et al., (2017) investigated silver nanoparticles decorated lipase-sensitive poly urethane micelles able to control *S. aureus* as an achievement to introduce new drug bead molecule.

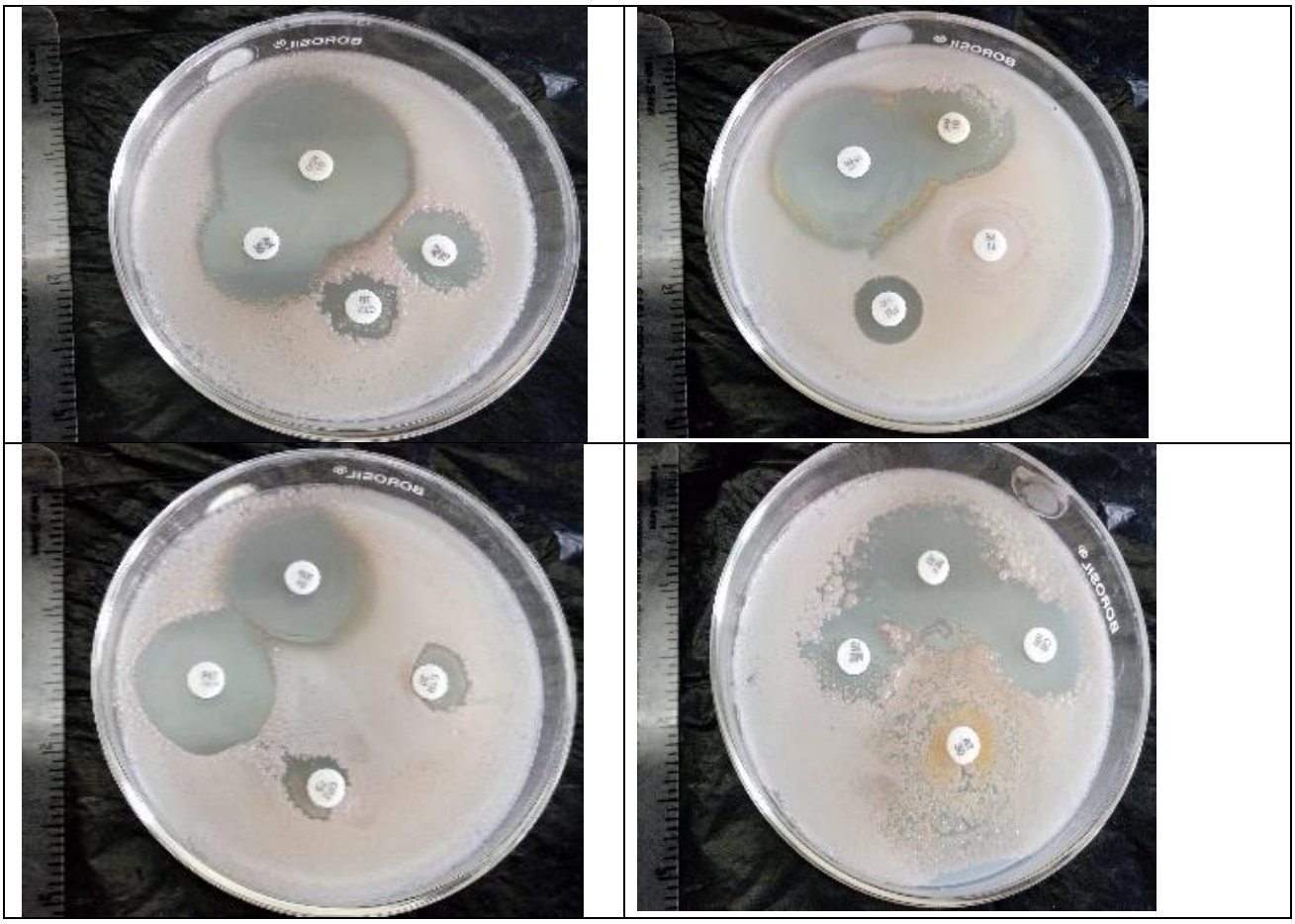


Figure 3. Antibiotic sensitivity against *S. aureus.*

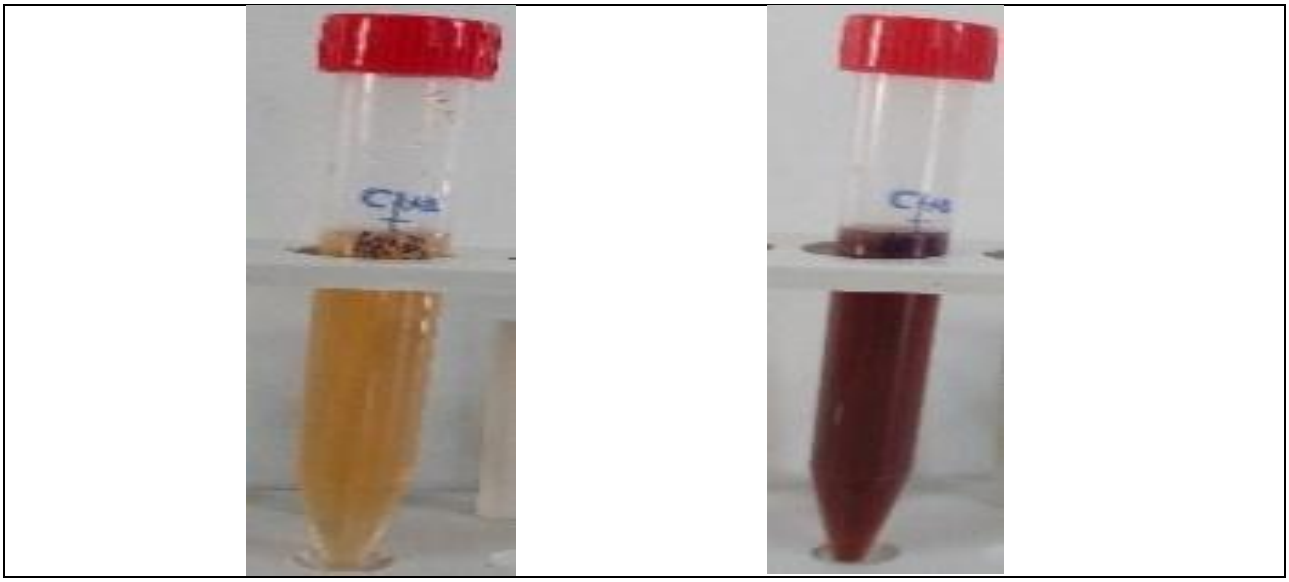


Figure 4a. clove+AgNo3 at 0hr.

Figure 4b. clove+AgNO3 after 24hrs

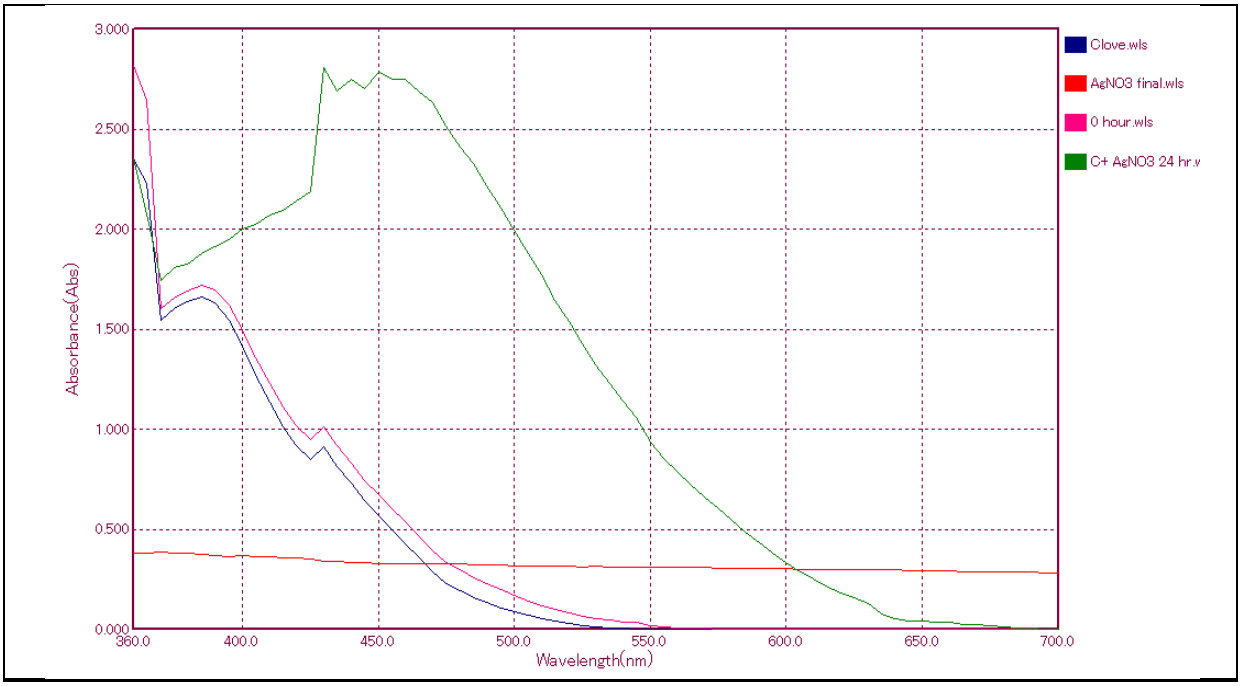


Figure 5.Comparision of absorption maxima of AgNO3, Clove, AGNPs at 0 and 24 hours.

Table 3. Antibiotic sensitivity against S. *aureus*.

Antibiotics	abbreviations	Zone of Inhibition (mm)									
		S. aureus 1	*S. aureus 2*	*S. aureus 3*	*S. aureus 4*	*S. aureus 5*	*S. aureus 6*	*S. aureus 7*	*S. aureus 8*	*S. aureus 9*	*S. aureus 10*
Azlocillin	AZ75	R	S	S	R	S	S	S	R	S	S
Doripenem	DOR10	S	R	R	S	S	R	S	S	R	S
Cefotaxime	CTX30	R	R	S	R	R	S	R	S	S	R
Amikacin	AK30	S	S	R	S	S	S	S	S	R	R
Ticarcilin	TI75	R	R	S	R	S	S	S	R	S	R
Imipenem	IPM10	R	R	S	S	R	S	S	S	R	S
Polymixin-B	PB300	S	S	R	R	S	S	S	S	S	S
Levofloxacin	LE5	S	R	R	S	S	R	S	R	S	R
Cefoperazone	CPZ75	R	S	S	R	S	S	S	S	R	R
Ceftriaxone	CTR30	S	R	R	R	R	S	R	S	S	R
Piperacillin/Tazobactam	PIT100/10	S	S	S	S	S	S	S	S	S	R
Norfloxacin	NX10	S	S	S	S	S	S	S	R	S	S
Aztreonam	AT30	S	R	S	R	R	S	S	S	S	S
Carbenicillin	CB100	S	S	S	S	S	R	S	S	R	S
Mezlocillin	MZ75	R	S	S	R	S	S	S	R	S	S
Netillin	NET30	S	S	S	S	S	R	S	S	S	S
	resistant	6	7	5	8	4	4	2	5	5	7
	sensitive	10	9	11	8	12	12	14	11	11	9
S: Sensitive; R: resistance as per CLSI chart											

Table 4. Antimicrobial activity of synthesized Ag NPs with respect to various parameters.

Bacteria	Zone of inhibition(mm)												
	Glucose concentration				Temperature				pH				
	1%(a)	2%(b)	3%(c)	4%(d)	20°C(a)	35°C(b)	45°C(c)	70°C(d)	3.5(a)	4.5(b)	5.5(c)	7.5(d)	9.5(e)
E. coli 1	NI	NI	NI	NI	NI	10	NI	10	11	NI	NI	12	18
E. coli 5	NI	NI	NI	NI	NI	9	NI	9	10	NI	NI	10	12
E. coli 7	NI	NI	NI	NI	NI	9	NI	8	10	NI	NI	NI	NI
S. aureus 2	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	14	18
S. aureus 4	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	12	31
S. aureus 7	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	10	20
*Note: NI= No Inhibition													

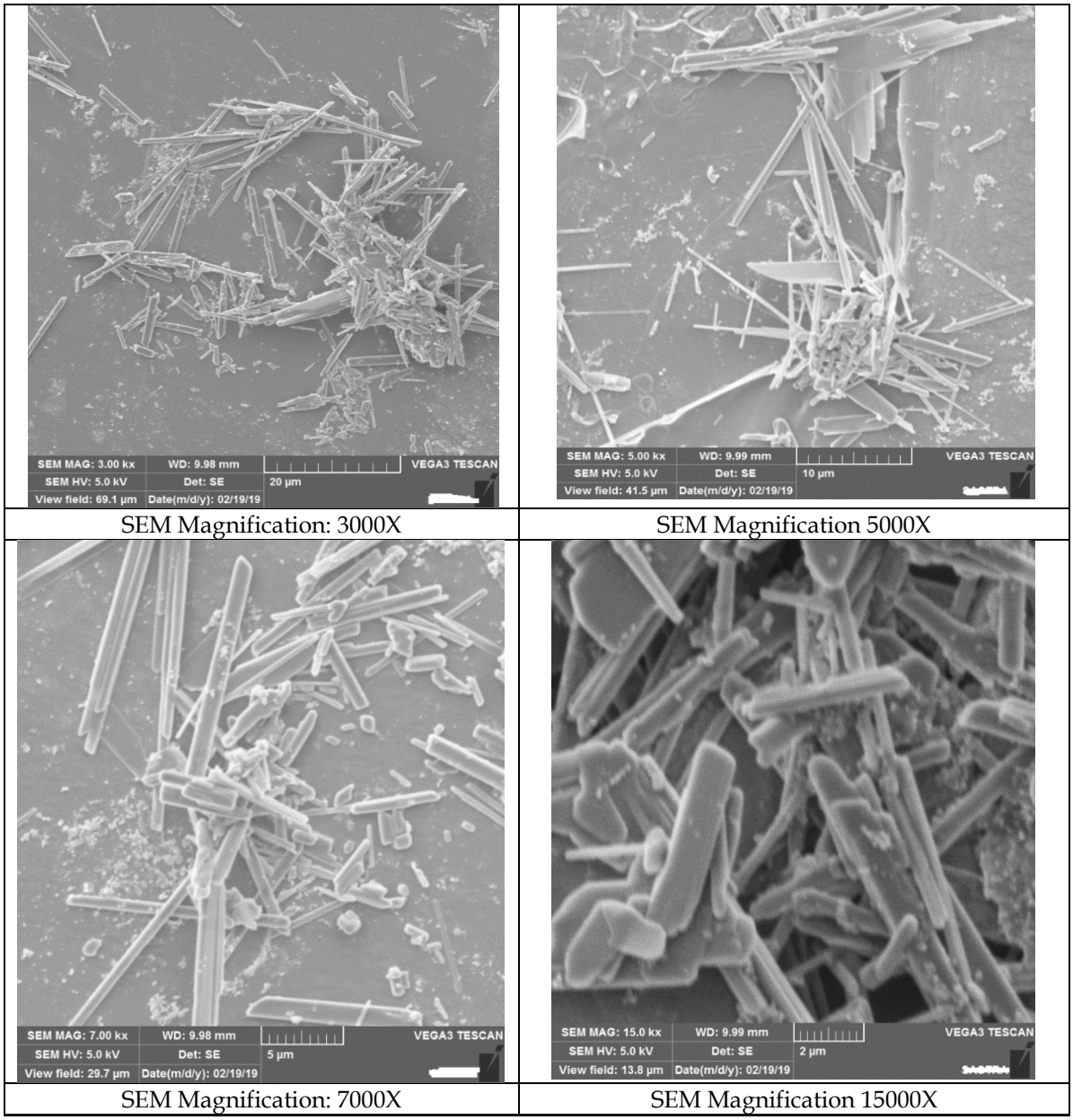


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Figure 6. Nanoparticle recognized as nanorods via SEM analysis of Clove bud set at 9.5 pH with variable resolution.

Here nanorods formed at basic pH (9.5) able to control MDR *S. aureus* as well as *E. coli* which was not evidenced significantly earlier.
Shaheen and Fouda (2018) reported a similar finding that silver nanorods could control multidrug-resistant cellsof Gram-positive and Gram-negative bacterial species. Xiang et al., (2017) also involved poly (lactic-co-glycolic acid) ZnOnano-rods/Ag nanoparticles hybrid coating to control *S. aureus* and *E. coli* with the presence of antibacterial features.

CONCLUSION

Humans wanted to survive in the world of ever-increasing pathogens. In an attempt, we trusted the potential of medicinal herb *S. aromaticum* flower bud to synthesize silver nanoparticles capable of controlling MDR *S. aureus* and *E. coli* prevalent in the human fluid as pathogens.
*S. aromaticum*AgNPs synthesized at pH 9.5 found to be nano-rods capable of controlling MDR *E. coli* and *S. aureus* by inhibiting their growth. Hence these nano-rods could be the future drug for better drug therapy for bacterial pathogenicity control.

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